Supporting information: Single Molecule Tracking on Supported Membranes with Arrays of Optical Nanoantennas

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Fabrication of Nanoantenna arrays:

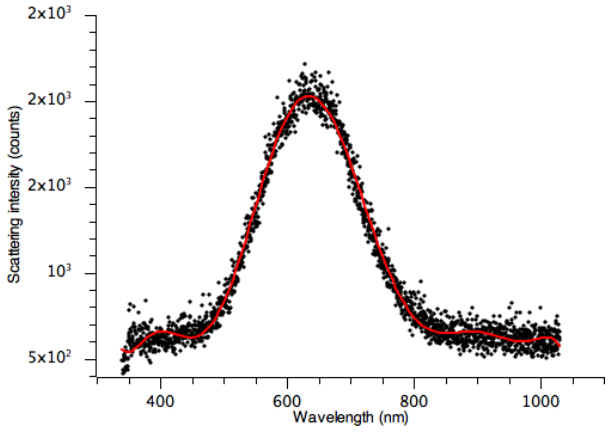
Colloid lithography: Polystyrene (PS) spheres were used for colloid lithography. (PS beads: 1.26 μm; PS beads: 0.69 μm; 2.6 % solution (by weight) in water, Bangs Laboratories, Inc., Fishers, USA). A colloid monolayer was prepared by sweeping the polystyrene beads over the surface of a freshly cleaned and activated glass slide at a velocity of 70 μm/min. Prior to usage, all glassware was cleaned with piranha solution (conc. H2SO4 / 30% H2O2 ; 3:1)

Plasma treatment: Particles were subject to plasma treatment using a Harrick PDC-32G, plasma etcher at a chamber pressure of 0.2mbar, a plasma power of 18 W. All experiments were performed using air plasma.

Metal evaporation: Gold was deposited by thermal evaporation (Veeco Instrument, Inc., Plainview, NY, USA) of 99.99% purity gold wire (Super Conductor Materials, Inc., Suffern, NY, USA) in a tungsten boat (R. D. Mathis Company, Long Beach, CA, USA) at 0.14-0.22 nm/sec (140-155 A electrode current) after ramping up current over the course of 2-4 minutes.

Nanoantenna substrate characterization: SEM measurements were performed using an Ultra 55 field emission scanning electron microscope (FE-SEM) operated at accelerating voltages of 2-5 kV (Zeiss SMT, Oberkochen, Germany).

Scattering spectra acquired by dark-field microscopy:



S2: *Dark-field spectra of the nanoantenna array: Scattering spectra of gold nanoantenna arrays fabricated with 0.69 µm polystyrene beads after 6 min plasma treatment.*

Single molecule tracking:

Protein preparation: SOScat from human SOS1 (residues 566–1049) with a single surface-exposed cysteine (point mutations C838A, C635A, C980S, E716C) and human H-Ras (residues 1-181, with point mutation C118S) were expressed and purified as previously described (Gureasko et al., 2008)[1]. SOScat and Ras were stored in (25 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 10% (w/v) glycerol and 2 mM Tris(2-carboxyethyl) phosphine (TCEP).

SOScat Labeling: SOScat was labeled site-specifically at E716C with Atto647N-maleimide (AttoTec). 1mg of reactive dye was dissolved in 100µL (25mM HEPES (pH 7.5), 100mM NaCl, 5mM MgCl2) and added in 10x molar excess to SOScat and allowed to react for 3 hours at 22 ^{o}C. Excess dye was removed by sequential gel filtration in two PD-10 size exclusion chromatography columns (GE Healthcare) into (50mM HEPES, 100mM NaCl, 5mM MgCl2). The degree of labeling was ~1.

Au-Nanopatterned Glass Substrate Cleaning: The patterned glass substrates were immersed in 1:1 IPA:ultrapure H2O overnight, sonicated in a bath sonicator for 5 minutes, followed by

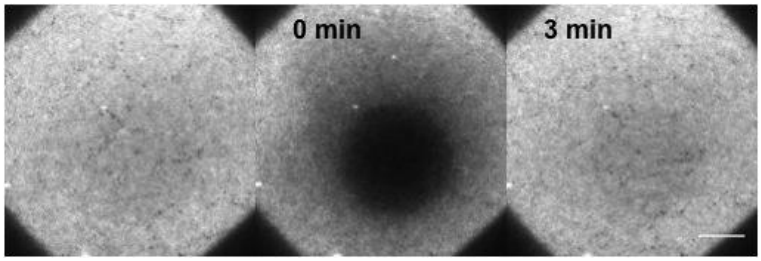
throrough rinsing with ultrapure water (MiliQ). The substrates were etched in warm Piranha solution (3:1, Sulphuric acid:hydrogen peroxide) for 2 minutes, followed by thorough rinsing with ultrapure water. The cleaned substrates were immediately assembled in flowcells (Bioptechs) for supported lipid bilayer deposition.

Preparation of Ras-functionalized Supported Lipid Bilayers: The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE) were purchased from Avanti Polar Lipids.

Maleimide-functionalized small unilamellar vesicles (SUVs) were prepared by drying a mixture of DOPC (83 mol%), DOPS (10 mol%) and MCC-PE (7 mol%) in chloroform on a rotary evaporator at 40^{o} C for 10 minutes. Dried lipid films were placed under a gentle nitrogen stream for 20 minutes and then re-suspended in degassed 1xPBS buffer (Cellgro, pH 7.4) at a lipid concentration of 1 mg/mL. Lipid suspensions were probe sonicated 2 times 35seconds in an ice-bath, under N2 flow, resulting in clear suspensions of SUVs. Ti particles were removed by 20 min centrifugation at 20.000xg at 4^{o} C. The SUVs were injected into flow-cells (Bioptechs), assembled with freshly cleaned glass substrates with Au nanogap patterns, and allowed to incubate for 30 minutes at 22^{o} C. Excess SUVs were rinsed away with 3 mL 1xPBS. Defects in the SLBs were blocked by 10 minutes incubation with casein, diluted 10 times from a saturated solution into 1x PBS.

Ras was desalted into 1xPBS using illustra NAP-5 columns. 1.5 mg/mL Ras was injected and allowed to couple to the MCC-PE in the bilayers for 2 hours and 45 minutes. Coupling reactions were terminated and excess maleimide-lipid quenched by addition of 5 mM beta-mercaptoethanol in 1xPBS, incubated for 10 minutes at 22^{o}C, followed by 3mL washing with 1xPBS. GDP was removed from SLB tethered Ras by incubation with 50mM EDTA (in 25mM HEPES, 100mM NaCl) for 20 minutes at 4^{o}C. EDTA was removed by washing with 3 mL (25mM HEPES,

100mM NaCl). Empty Ras was loaded with 10 µM Atto488-GTP (Jena Bioscience) in (25 mM HEPES, 100mM NaCl, 5 mM MgCl2) for 2 h at 4 °C. Unbound fluorescent nucleotide was washed away with 3 mL (25 mM HEPES, 100mM NaCl, 5 mM MgCl2). Fluorescence recovery showed that labeled nucleotides on Ras were laterally mobile on the nanogap array (Figure S2).



S2: *FRAP measured on a nanoantenna substrate.*

Microscopy: 200µL SOScat-Atto647N at 400pM (25 mM HEPES, 100mM NaCl, 5 mM MgCl2) was injected through the samples (2.3 mL/min), resulting in single molecules of SOScat-Atto647N bound to Ras-GTP-Atto488 diffusing on the nanopatterned substrates. Total internal reflection (TIR) microscopy for single-molecule tracking was done on a Nikon Eclipse Ti microscope equipped with an Andor emCCD camera. Image acquisition time was set to 150 ms.

[1] Gureasko, J. *et al.* Membrane-dependent signal integration by the Ras activator Son of sevenless. *Nature Structural & Molecular Biology* **15**, 452 - 461 (2008).

SERS imaging:

Raman microscope setup: Two-dimensional spectral maps were acquired with a confocal Raman microscope (WITec, alpha300 S, fiber/pinhole diameter = 100 µm), which is equipped with a piezoelectric scan stage. In order to obtain high-quality and high-resolution images a 100x microscope objective (Nikon, NA = 0.95) and a laser in the visible wavelength range (λ = 532 nm) were used in the measurements. The linearly polarized laser light was focused with a nearly diffraction-limited spot size onto the samples and the Raman light was detected by a CCD camera (Andor, DV401 BV) behind a grating (600 grooves mm-1) spectrometer (WITec, UHTS 300) with a spectral resolution of 3 cm^{-1}. The laser power on the samples was approximately 750 µW.

Sample areas of interest were mapped by raster scanning in 167-nm steps with an integration time of 130 ms per spectrum. The WITec Project software (version 1.94) was used for spectral and image processing and analysis. Chemical images were computed from the two-dimensional spectral maps by integrating the intensity over a defined wavenumber range in the baseline-corrected Raman spectra.

Calculation of the enhancement factor EF: The resolution of the objective in the xy plane is defined by the Rayleigh limit to be:

$$r_{xy} = \frac{0{,}61 * \lambda}{NA}$$

For λ = 532 nm and NA = 0.9, r_{xy} was calculated to be 360 nm. Between one and three gaps can be measured at the same time by the confocal laser spot.

The enhancement of the Raman signal from the bowtie array was directly compared with the Raman signal measured from a thin gold film of the same sample. Both, the SERS spectra and the reference spectra were thus taken from a sample that was prepared under identical experimental conditions (experimental setup, labeling chemistry, surface roughness of the gold film etc.). Since the surface density of molecules on the metal can be assumed to be identical for both, SERS spectra and reference spectra, the EF was calculated according to:

$$EF = \frac{I_{SERS} / A_{nanogap}}{I_{Raman} / A_{Laserspot}}$$

$A_{nanogap}$ was derived from the field enhancement calculated by finite element analysis. A radius of curvature of 10 nm was assumed for the nanoantenna tips.